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Calibrated Chemiluminescence Standards

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Summary: The aim of this project was to provide an absolute photometric calibration for an underwater photometer in use at the Naval Oceans Systems Center in San Diego. This was made by using as a reference light standard, the chemiluminescence reaction of luminol. The quantum yield of this chemiluminescence is known. Since it was not possible to conveniently initiate the chemiluminescence reaction within the underwater photometer, it was first necessary to develop a liquid light standard which could be used for substitution. A recipe was developed for making a suspension of luminous bacteria that would emit light at a constant rate for about 30 minutes. Its photon emission rate could be easily determined using a second photometer calibrated against luminol.

In the second part of the project an attempt was made to verify the published quantum yield of the luminol chemiluminescence using the new silicon photodiode absolute photometer now available at the National Bureau of Standards. The results show satisfactory agreement with the previous measurements but the measurements are still preliminary and significant optical problems still need to be resolved.

Report on Calibration of Photomultiplier Photometers at the

Naval Ocean Systems Center - San Diego.

Technique: A dilution of marine bacteria, Photobacterium phosphoreum strain A-13 into filtered sea water gave stable emission over a period of >30 minutes.

The emission rate of these bacteria, a 1 ml sample was measured absolutely using a bioluminescence field photometer, property of the Bioluminescence Group, University of Georgia. This photometer hereafter known as YB (yellow box) was field calibrated using a radioactive standard of C-14 POPOP. POPOP has a somewhat different emission spectrum from the A-13 bacteria and previous experiments on YB had shown that for a 1 ml sample.

1 photon count of bacteria

= (1.26 ± 0.07) photon/count relative to the POPOP standard.

Thus measured counts/ml. were measured as bacterial counts = YB counts X 1.26.

Three photometer systems were calibrated. These were A, a vial photometer with a 5.6 ml (measured volume) vial, B a flow photometer with a 25 ml chamber, and C a submersible flow photometer with a 25 ml chamber.

Photometer A

Photometer A had a vial of 5.6 ml measured volume and a ND 2 filter in front of one photomultiplier and a UV filter in front of the other. No significant counts were observed for the UV PM.

Calibration factor C = 1.26 X 5.6 = 7.056.

I counts/100 sec.	YB hv	$F = CXYB/_I$
		dark counts negligible
9.4×10^6	9.5×10^8	713
9.17×10^6	9.2×10^8	722
8.87×10^6	9.0×10^8	716
8.53×10^6	8.8×10^8	728

$\sim$10 X dilu. dark counts 3.6×10^4 = D

I,I-D

6.12/5.76 X 10^5	5.9 X 10^7	723
5.83/5.47 X 10^5	5.6 X 10^7	722
5.50/5.14 X 10^5	5.3 X 10^7	728

The more conc. and dilute experiments are not significantly different and yield a mean of

$$F = (720 \pm 8) \text{ hv/count,}$$

This

is a 1.1% std. deviation.

Photometer B

These measurements were carried out on the morning of Friday the 14th. Measurements of the previous afternoon were discarded since the photometer battery was indicating discharged. PM in use had an ND 2 filter incorporated.

YB had 1.0 ml. volume and the sample chamber a volume of 25 ml.

Thus 1 count relative to POPOP, YB, is equivalent to 25 X 1.26 = 31.5 counts in the system chamber.

$$\text{i.e. } C = 31.5$$

The counts as measured in the chamber decreased slowly with time. 100 second accumulations of counts were measured with time and the intensity at t = 0, I_0 was obtained by extrapolation of a logarithmic line.

The dark counts were assumed to be negligible.

(i)

I	YB	t(s) (at center of 100 sec time window)
1.232 X 10^7	7.0 X 10^8	90
1.197 X 10^7	7.3 X 10^8	160
1.083 X 10^7	6.97X 10^8	310
9.50 X 10^6	6.84X 10^8	420
	Mean 7.0 X 10^8	

$I_0 = 1.303 \times 10^7$ counts,   for $F = \dfrac{YB \times C}{I_0}$

$$F = 1698 \text{ photons/count.}$$

(ii)

I	YB	t
1.056×10^7	4.76×10^8	88
9.78×10^6	5.0×10^8	214
9.12×10^6	5.04×10^8	326
8.33×10^6	4.81×10^8	442
$I_o = 1.116 \times 10^7$	$\overline{YB} = 5.0 \times 10^8$	

i.e. F = 1411

(iii) System B.

I	YB	t
1.306×10^7	5.65×10^8	82
1.171×10^7	5.68×10^8	191
1.0895×10^7	5.71×10^8	303
1.0516×10^7	5.77×10^8	415
9.40×10^6	5.70×10^8	537
$I_o = 1.37 \times 10^7$	$\overline{YB} = 5.7 \times 10^8$	

and F = 1311

(iv)

I	YB	t
1.0803×10^7	5.2×10^8	74
9.935×10^6	5.2×10^8	188
9.026×10^6	5.02×10^8	301
8.317×10^6	4.92×10^8	415
$I_o = 1.139 \times 10^7$	$\overline{YB} = 5.2 \times 10^8$	

and F = 1438

(v)	I	YB	t
	1.275×10^7	5.14×10^8	66
	1.1395×10^7	5.20×10^8	177
	1.067×10^7	5.26×10^6	290
	9.254×10^6	5.26×10^8	409
	$I_o = 1.35 \times 10^7$	$\overline{YB} = 5.26 \times 10^8$	

$$\text{and } F = 1227$$

collecting F values,

(i)   1698 disregarding (1) $\overline{F} = (1347 \pm 97)$ hv/count.

(ii) 1411

(iii) 1311

(iv) 1438, this is a 7% std. deviation

(v)   1227

Photometer C

Submersible photometer "Black hole". As with B, sample volume was 25 ml and C = 31.5. The decay of the bacteria light standard in this chamber, while less serious than that of B, was allowed for by the same back extrapolation method.

Dark counts 2.4×10^5

(i)	I/I-D	YB	t(sec)
	$8.22/8.205 \times 10^6$	3.83×10^8	68
	$7.487/7.463 \times 10^6$	4.08×10^8	178
	$7.264/7.240 \times 10^6$	3.98×10^8	288
	$5.3916/5.368 \times 10^6$	3.94×10^8	395
	$5.658/5.634 \times 10^6$	3.96	506
	$I_o = 8.67 \times 10^6$	$\overline{YB} = 4 \times 10^8$	

$$F = \frac{C \times YB}{I_o} = 1453. \text{ NB}$$

Black hole power supply unsteady, replaced with another power supply for later experiments.

(ii)

$7.611/7.587 \times 10^6$	2.84×10^8	104
$7.454/7.430 \times 10^6$	3.08×10^8	214
$7.286/7.262 \times 10^6$	3.10×10^8	340
$7.098/7.074 \times 10^6$	3.10×10^8	448
$I_o = 7.72 \times 10^6$	$\overline{YB} = 3.1 \times 10^6$	

$$F = 1265$$

(iii) System C.

$11.971/11.947 \times 10^6$	4.5×10^8	61
$11.094/11.070 \times 10^6$	4.45×10^8	172
$11.035/11.011 \times 10^6$	4.52×10^8	284
$10.816/10.792 \times 10^6$	4.40×10^8	396
$10.511/10.487 \times 10^6$	4.35×10^8	507
$10.176/10.152 \times 10^6$	4.18×10^8	615
$I_o = 1.206 \times 10^7$	$\overline{YB} = 4.50 \times 10^8$	

$$F = 1175$$

(iv) Dark counts $= 2.9 \times 10^4$

$9.766/9.740 \times 10^6$	3.61×10^8	60
$9.261/9.235 \times 10^6$	3.88×10^8	167
$8.972/8.946 \times 10^6$	3.76×10^8	275
$8.774/8.748 \times 10^6$	3.74×10^8	386
$8.598/8.572 \times 10^6$	3.66×10^8	495
$8.409/8.383 \times 10^6$	3.63×10^8	605
$I_o = 9.90 \times 10^6$	$\overline{YB} = 3.88 \times 10^8$	

$$F = 1235$$

(v)   Dark counts = 2.9×10^4

$8.088/8.059 \times 10^6$	3.25×10^8	61
$8.039/8.009 \times 10^6$	3.32×10^8	173
$7.879/7.849 \times 10^6$	3.33×10^8	285
$7.709/7.680 \times 10^6$	3.31×10^8	395
$7.515/7.486 \times 10^6$	3.26×10^8	503
$7.292/7.263 \times 10^6$	3.27×10^8	612
$I_o = 8.27 \times 10^6$	$\overline{YB} = 3.33 \times 10^6$	

$$F = 1268$$

Collecting F values

(i) 1453

(ii) 1265

$$\overline{F} = (1236 \pm 43) \text{ hv/count}$$

(iii) 1175

(iv) 1235

(v) 1268, this is a std. deviation of 3.5%

The precision of calibration of the yellow box $\overline{YB}$ was 6%. Including this the final results are:

Photometer A, $F = 720 \pm 50$ hv/count

Photometer B, $F = 1350 \pm 190$ hv/count

Photometer C, $F = 1240 \pm 130$ hv/count

These sensitivities are less than the F values derived from known solid angles and the manufacturer's photocathode sensitivity curve. The low values may in part be accounted for by the photomultiplier sensitivity being less than its peak value over much of the range of bioluminescence emission, and variations in overall photomultiplier sensitivity.

Preliminary Report on Calibration of the Luminol Standard

Chemiluminescence with a Standard Silicon-detector

Radiometer at the National Bureau of Standards.

Objectives:

This first visit in May was carried out with the purpose of determining

the optimum experimental conditions; a later trip in July was used to perform

more definitive experiments. This work was carried out in collaboration with

Dr. Edward F. Zaleswski, N.B.S. who provided and operated the N.B.S. silicon

diode absolute radiometer.

Materials:

A solution of luminol (Aldrich), unpurified, in pH 11.6 $NaHCO_3$ was prepared.

It possessed an optical density of 36.6 at 347 nm, concentration by weight 5.67 mM.

This was stored in a brown bottle and carried to the N.B.S. 1 ml sample of luminol

in a clear bottom fluorescence cuvette was used and the reaction initiated by

addition of 10 µl of 30% H_2O_2 and 5 µl of 10 mg/ml of horseradish peroxidase in

pH 11.6 buffer.

Apparatus:

The silicon detector radiometer was arranged so that it viewed upwards through

the bottom of a 1 cm fluorescence cuvette. The cuvette sat on top of a pair of

vertically stacked apertures of 0.5 sq. cm.

This geometry is analogous to the problem of heat flow between two apertures

and is capable of solution. Further details of the analysis of this problem will

be forthcoming from Dr. Zaleswski.

The cuvette and detector were surrounded by a cylinder constructed of black

cord with a loose fitting top. This did not prove to be light tight so that the

experiments had to be carried in total darkness. The output from the radiometer was amplified using the N.B.S. supplied current amplifier and fed to the input of a sensitive strip chart recorder. The strip chart output was integrated by means of a planimeter.

Results:

Reaction of a neat 5.67 mM luminol solution showed that adequate light was available in the initial stages of the reaction. (This reaction was not carried to completion because of excessive time required). Accordingly the stock solution was diluted ~100 X (The type of pipette used for the dilution was such that approx. 0.12 rather than 0.10 ml was diluted to 10.0 ml; so that the concentration was uncertain).

These yielded for the first 3 experiments an integrated current output of

1) 4.52 X 10^{21}

2) 4.45 X 10^{21}

3) 4.02 X 10^{21} electrons/mole

A further experiment was carried out using a 10X dilution of this solution (accurate this time) and yielded

4) 3.94 X 10^{21} electrons/mole for 5.67 μM luminol.

The diluted solutions were stored overnight in darkness in clear glass flasks. On the second day it was determined that 100X dilution yielded 1) 3.31 X 10^{21} electrons/mole. This is considerably lower than the day before and may indicate luminol photo-decomposition.

A fresh solution 0.0Ω 0.1 ml of 5.67 μM stock to 23 ml was prepared, yielding

2) 2.65 X 10^{21} electrons/mole

3) 2.76 X 10^{21} electrons/mole

4) 2.82 X 10^{21} electrons/mole

5) 2.88 X 10^{21} electrons/mole

It is notable that experiments 3) and 4) were carried out with a spacer separating the two apertures so that in principle only about 1/2 as much is transmitted from sample to detector. They yield results negligibly different from the others suggesting that the assumptions regarding geometry are reasonable.

Conversion of the electrons/mole to photons per mole requires knowledge of the silicon detector quantum efficiency averaged over the luminol emission band. Approximating this to be 0.53, the value of the quantum efficiency at the 420 nm maximum of luminol, this suggests for the final (250X   a set) that the luminol quantum efficiency of chemiluminscence in 0.9%.

This is more than close enough to the literature value (   ), at least for a first attempt.

Conclusions:

1) The problem is feasible.

2) A convenient luminol concentration is 10-30 µM at which concentration the reaction takes 45-60 minutes to completion.

3) The reaction may be readily initiated by adding µl amounts of H_2O_2 and catalyst to 1 ml of luminol solution, thus obviating corrections due to volume changes.

4) The sensitivity of the detector is such that the base initiated luminol reaction in DMSO may be studied.

Second Visit

Measurements of these reactions were repeated under more optimized conditions and the results found to be in satisfactory agreement with the first set of experiments. The data has not been analyzed in detail as of the time of writing this Report.

Attention was also given to the optical geometry of the experiment. Accurate determination of this is a major source of uncertainty (±30%) for these photometric measurements. Preliminary results indicate that the assumptions made in the calculation of this geometry are approximately valid. This problem however still requires more attention before confidence can be given to its solution.

Conclusion

The measurement of the chemiluminescence quantum yield of the luminol reaction in aqueous solution using the NBS Standard Photometer, is in satisfactory agreement with the previously published value, 1•2%, of Lee and Seliger. A large uncertainty arises from the optical geometrical correction. Further work will be able to solve this problem. The experiments will be able to be done also for the DMSO reaction, and also using more purified luminol samples. An integrating sphere geometry should also be tried since this should reduce or eliminate the optical geometric uncertainty.